

# Cell membrane damage induced by phenolic acids on wine lactic acid bacteria

F.M. Campos<sup>\*</sup>, J.A. Couto, A.R. Figueiredo, I.V. Tóth, A.O.S.S. Rangel, T.A. Hogg

CBQF/Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072 Porto, Portugal

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Biocides

## A B S T R A C T

The aim of this work was to investigate the effect of phenolic acids on cell membrane permeability of lactic acid bacteria from wine. Several phenolic acids were tested for their effects on the cell membrane of *Oenococcus oeni* and *Lactobacillus hilgardii* by measuring potassium and phosphate efflux, proton influx and by assessing culture viability employing a fluorescence technique based on membrane integrity. The experimental results indicate that hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids) induce greater ion leakages and higher proton influx than hydroxybenzoic acids (*p*-hydroxybenzoic, protocatechuic, gallic, vanillic, and syringic acids). Among the hydroxycinnamic acids, *p*-coumaric acid showed the strongest effect. Moreover, the exposure of cells to phenolic acids caused a significant decrease in cell culture viability, as measured by the fluorescence assay, in both tested strains. The results agree with previous results obtained in growth experiments with the same strains. Generally, phenolic acids increased the cell membrane permeability in lactic acid bacteria from wine. The different effects of phenolic acids on membrane permeability could be related to differences in their structure and lipophilic character.

## Introduction

Wine can be a challenging environment for the growth of many bacterial species due to its intrinsic characteristics (relatively high ethanol content, low pH and nutrient availability) and to the presence of antimicrobial agents such as sulphur dioxide. However, some bacterial strains, most notably some species of lactic acid bacteria (LAB), are able to thrive in wine and ultimately modify its composition. Depending on the strain, and on the moment they multiply, LAB may be beneficial or detrimental to wine quality (Lonvaud-Funel, 1999).

LAB are responsible for the occurrence of malolactic fermentation (MLF), a secondary fermentation which is considered to be beneficial in most red wines and in some white wines (Liu, 2002). *Oenococcus oeni* is considered to be the most common LAB that conducts this process (Cavin et al., 1993; Nielsen et al., 1996; van Vuuren and Dicks, 1993) and selected strains of this species are nowadays widely used as starter cultures in the wine industry. Other LAB species, like *Lactobacillus hilgardii* (which is commonly found in spoiled fortified wines) can cause wine deterioration, increasing its volatile acidity and producing off-flavours (Couto and Hogg, 1994; de Revel et al., 1994).

The cell (or cytoplasmic) membrane of bacteria normally acts as a diffusion barrier between the cytoplasm and the extracellular medium. The integrity of this membrane is fundamental to maintain the chemiosmotic balance which is necessary for the membrane-associated energetic metabolism of LAB (Konings, 2002). Under the unfavourable conditions of the wine environment, this metabolism may be critical to the survival of these bacteria.

Some wine components (ethanol, pH and sulphites) are known to affect the physical and chemical properties of the cytoplasmic membrane of LAB (Spano and Massa, 2006).

Despite extensive research having been done concerning the effect of ethanol on the cytoplasmic membrane of *O. oeni* (Chu-Ky et al., 2005; da Silveira et al., 2002, 2003, 2004; Teixeira et al., 2002), and also some on *L. hilgardii* (Couto et al., 1996, 1997), little work has been done concerning the effect of wine phenolic compounds on this cellular component.

It has been empirically known for years that the phenolic content of grapes and wines can affect the rate of malolactic fermentation. The phenolic composition of wines is much diversified and includes phenolic (hydroxybenzoic and hydroxycinnamic) acids in concentrations up to 200 mg/L (Reguant et al., 2000). Despite their structural similarities (see Fig. 1), phenolic acids may have positive or negative effects on wine lactic bacteria depending on the nature and concentration of the compound and on the bacterial strain (García-Ruiz et al., 2008; Reguant et al., 2000). Several authors reported the

<sup>\*</sup> Corresponding author. Tel.: +351 225580001; fax: +351 225090351.  
E-mail address: fmcampos@mail.esb.ucp.pt (F.M. Campos).

Group	Chemical structure	Name
Hydroxybenzoic acids		$R'=R=H$ – <i>p</i> -Hydroxybenzoic acid
		$R'=H$ ; $R=OH$ – Protocatechuic acid
		$R'=R=OH$ – Gallic acid
		$R'=H$ ; $R=OCH_3$ – Vanillic acid
		$R'=R=OCH_3$ – Syringic acid
Hydroxycinnamic acids		$R'=R=H$ – <i>p</i> -Coumaric acid
		$R'=H$ ; $R=OH$ – Caffeic acid
		$R'=H$ ; $R=OCH_3$ – Ferulic acid

Fig. 1. Structural formulae of phenolic acids used in this work.

stimulatory effects of gallic acid at low concentrations on the growth and malolactic activity of LAB (Alberto et al., 2001, 2004; Vivas et al., 1997). Conversely, other authors have found that at high concentrations hydroxycinnamic acids can have a negative effect on the same parameters (Campos et al., 2003; Reguant et al., 2000; Salih et al., 2000; Stead, 1993). Phenolic acids are also known to delay the metabolism of sugars and citric acid by wine LAB (Campos et al., 2009; Rozès et al., 2003).

LAB can interact with wine phenolic acids in different ways. It has been reported that strains with cinnamoyl esterase activity can hydrolyse caftaric and coutaric acids during MLF increasing the concentration of the corresponding free hydroxycinnamic acids (Hernández et al., 2007a,b). Other authors have demonstrated that some LAB are able to produce volatile phenols (vinylphenols and ethylphenols) from the metabolism of hydroxycinnamic acids (Cavin et al., 1993; Chatonnet et al., 1992, 1995; Couto et al., 2006; Landete et al., 2007; Rodríguez et al., 2008b; van Beek and Priest, 2000). Further works have shown that some strains of *Lactobacillus plantarum* are able to degrade hydroxybenzoic acids producing some compounds like pyrogallol and catechol which might be beneficial for the growth and metabolism of this bacterium (Alberto et al., 2004; Landete et al., 2007; Rodríguez et al., 2008a).

Phenolic compounds are known to have membrane-active properties against microorganisms causing leakage of cell constituents (Johnston et al., 2003). These compounds may diffuse through the cytoplasmic membrane increasing its permeability. Following their influx there is usually a leakage of bacterial cell constituents including proteins, nucleic acids, and inorganic ions such as potassium or phosphate. The measurement of the efflux rates of these metabolites (using potentiometric or spectrophotometric methods) has been used for some time to monitor cell membrane damage (Denyer and Hugo, 1991).

The determination of cell membrane damage caused by phenolic compounds by spectrophotometric methods is especially challenging since these compounds strongly absorb UV radiation in the 260–280 nm range (which is normally used to assess efflux of cellular components such as proteins and nucleic acids) whilst other phenolics are coloured and thus may interfere with colour-based spectrophotometric determinations.

The potassium efflux approach has been used by several authors to assess the effect of different biocides on bacteria, including LAB (Al-Adham et al., 1998; Heipieper et al., 1991; Hong and Pyun, 2001; Johnston et al., 2003; Ohmizo et al., 2004). Direct potentiometry (using ion-specific electrodes) is a particularly convenient method for the measurement of this efflux since it enables a continuous monitoring of the extracellular medium without the need of separation of the cells.

Other authors have used extracellular pH measurement to monitor proton influx in *O. oeni* cell suspensions exposed to ethanol (da Silveira et al., 2002).

The use of fluorimetric techniques and fluorescence microscopy for assessing the viability of microorganisms has been increasing since they provide fast and sensitive results together with the ability to detect viable but non-culturable cells (Breeuwer and Abee, 2000). The discrimination of viable and non-viable cells is normally based on cell membrane integrity, which allows the selective influx of fluorescent dyes and the differential staining of the cells. Fluorescence viability kits have been used by many authors to monitor cell viability of LAB (Alakomi et al., 2005; Corich et al., 2004; Couto and Hogg, 1999; Maukonen et al., 2006; Moreno et al., 2006). Some authors have used a multiple-method approach to study the effect of biocides on bacterial membranes (Fitzgerald et al., 2004; Johnston et al., 2003). This type of approach seems to be more appropriate than a single-method approach since it allows for a greater confidence on the obtained results.

In this work, we used different instrumental techniques (potentiometry, spectrophotometry and fluorimetry) to evaluate the effect of wine phenolic acids on cell membrane integrity of LAB strains associated with wine.

## Materials and methods

### Bacterial strains and growth conditions

*L. hilgardii* strain 5, isolated from Port by Couto and Hogg (1994), from the ESBUCP (Escola Superior de Biotecnologia da Universidade Católica Portuguesa, Porto, Portugal) culture collection and *O. oeni* commercial strain Viniflora Oenos (VF) from Christian Hansen (Hrevdre, Denmark) were used. *L. hilgardii* strain 5 was chosen for being taxonomically representative of the predominant ethanol-tolerant species found in Port wine (Couto, 1996; Couto and Hogg, 1994).

The liquid growth medium used in this experiment (MRS/TJ) was a mixture (50:50) of two commercial media: MRS (de Man, Rogosa and Sharpe) from Biokar Diagnostics (Beauvais, France) and TJ (Tomato Juice broth) from Difco (Detroit, MI). The initial pH was adjusted to 4.5 with a concentrated (6M) hydrochloric acid solution before sterilizing. After sterilization (121 °C, 15 min), ethanol (99.5% v/v) was added to the medium to obtain a final concentration of 5% (v/v) ethanol.

### Preparation of bacterial suspensions

Bacteria were cultivated to late exponential phase in MRS/TJ (4 days at 25 °C in aerobiosis) and then centrifuged (10 min, 3000×g)

and resuspended in sterile phosphate buffer solution ( $\text{NaH}_2\text{PO}_4$  0.1 M, pH 4.5), for the potassium efflux and pH measurement experiments, or in sterile ultrapure water for the phosphate efflux and fluorescence viability experiments.

Cells were centrifuged again (10 min, 3000  $\times$ g) and the obtained cell pellet was weighed (in an analytical scale) before being resuspended using phosphate buffer or ultrapure water (as described previously) to 10% of the original volume of culture (thus obtaining a 10-fold increase in cell concentration). Average wet weight values of the cellular pellets were 0.65 g for *L. hilgardii* 5 and 0.41 g for *O. oeni* VF with relative standard deviations (RSD) of less than 15% and 16%, respectively.

#### Preparation of phenolic acid solutions

Concentrated (10-fold) solutions of hydroxybenzoic acids (*p*-hydroxybenzoic, protocatechuic, gallic, vanillic, and syringic acids) and hydroxycinnamic acids (*p*-coumaric, caffeic and ferulic acids) were prepared from the pure compounds by dissolving the appropriate amounts in ethanol (99.5% v/v). All compounds had a purity of at least 98% and were obtained from Sigma-Aldrich (Steinheim, Germany). These solutions were prepared immediately before use to minimize oxidation of the phenolic compounds.

#### Potassium afflux and extracellular pH measurement

Potassium leakage from cells was measured continuously by determining the extracellular potassium concentration of the cellular suspensions using a potassium ion-sensitive electrode (without internal reference solution) immersed in the cellular suspensions. The sensor system was constituted by a mixture of valinomycin, tetrakis(4-chlorophenyl)borate and dioctyl-sebacate (Niemán and Horvai, 1985). An Orion 900200 double junction electrode from Thermo Orion (Waltham, MA) was used as a reference electrode and the outer compartment of this electrode was filled with a 0.1 M  $\text{NaH}_2\text{PO}_4$  solution. The electrode was calibrated using standard solutions with known potassium concentrations (in the range of  $10^{-4}$ – $10^{-2}$  M) prepared in 0.1 M  $\text{NaH}_2\text{PO}_4$ .

Extracellular pH was monitored using a Crison (Barcelona, Spain) combined glass pH electrode. Proton influx was expressed as the rate of decrease of the concentration of extracellular protons, as described by da Silveira et al. (2002).

Potentiometric measurements were carried out using two Crison model 2002 voltmeters connected to a dual channel Kipp & Zonen BD 112 chart recorder (Delft, Netherlands).

Twenty millilitres of fresh cellular suspensions in 0.1 M  $\text{NaH}_2\text{PO}_4$  was placed in a 25 mL beaker on a magnetic stirrer and the electrodes were immersed in the suspensions. After stabilisation of the signals, 2.50 mL of the corresponding phenolic acid solution (at 20 g/L in ethanol) was added and the  $\text{K}^+$  and  $\text{H}^+$  potentiometric signals were recorded for a period of 5 min.

The effect of the addition of different phenolic acids and of the increasing concentrations of *p*-coumaric acid on the rate of potassium efflux and proton influx of the bacterial suspensions was evaluated for both strains. Each experiment was repeated to verify the obtained results.

The repeatability of both potentiometric determinations was determined by repeating the control assay at the beginning and at the end of each experiment. The reproducibility was also determined by comparing the results of independent experiments. The repeatability values for potassium efflux and proton influx measurements were estimated to be better than 10% ( $\text{RSD} \leq 10\%$ ;  $n = 2$ ) for both strains. The reproducibilities of potassium efflux and proton influx measurements were found to be better than 14% ( $\text{RSD} \leq 14\%$ ;  $n = 3$ ) and 20% ( $\text{RSD} \leq 20\%$ ;  $n = 3$ ), respectively, for both strains.

#### Phosphate efflux measurement

Phenolic acid solutions were added to fresh bacterial suspensions (in ultrapure sterilized water) in order to obtain a final concentration of 2.2 g/L and 11% (v/v) ethanol. Samples were collected at regular intervals for 5 min and micro-filtered (using sterile syringe filters with 0.45  $\mu\text{m}$  pore size). Then, the filtered samples were injected in a Flow Injection (FIA) system assembled for phosphate determination as described in Torres et al. (2007) with a modification to allow the direct injection of the filtrates. Gallic acid was not tested in these experiments since it was found to interfere with the phosphate detection reaction (Torres et al., 2007). Calibration was performed with  $\text{NaH}_2\text{PO}_4$  standard solutions in ultrapure water with concentrations in the range  $10^{-6}$ – $10^{-5}$  M, expressed as molar phosphorus concentration. The experiment was repeated for each bacterial strain to verify the obtained results. The repeatability and reproducibility of these determinations were found to be better than 21% ( $\text{RSD} \leq 21\%$ ;  $n = 2$ ) and 25% ( $\text{RSD} \leq 25\%$ ;  $n = 4$ ) for both strains.

#### Fluorimetric detection of cell membrane injury

The commercial bacterial viability kit LIVE/DEAD® BacLight™ from Molecular Probes (Eugene, OR) was used in this work. This kit is composed of two nucleic-acid binding fluorochromes: SYTO 9™, a green fluorescent dye which penetrates both viable and non-viable cells, and propidium iodide, a red fluorescent dye which penetrates non-viable cells only and quenches the fluorescence of SYTO 9™. Thus, bacteria with intact cell membranes fluoresce green while those with damaged membranes fluoresce red. The excitation and emission maxima of the fluorescent dyes are: 480 and 500 nm, respectively, for SYTO 9™ and 490 and 635 nm for propidium iodide.

Cell suspensions of lactic bacteria (in ultrapure water) were exposed to chemical stress with 500 mg/L of phenolic acids and 11% (v/v) ethanol for 10 min. Subsequently, a 1 mL sample of the cell suspension was quickly centrifuged (1 min, 11000  $\times$ g), washed with ultrapure water and mixed with 3  $\mu\text{L}$  of a 50:50 propidium iodide/SYTO 9™ mixture. The dyed suspension was kept in the dark for 10 min. The fluorescence of viable cells was measured using a Shimadzu (Duisburg, Germany) RF-1501 Fluorescence Spectrophotometer, using 480 nm as the excitation wavelength and 500 nm as the emission wavelength. Dilutions with ultrapure water were performed when the fluorescence intensity exceeded the upper limit of the fluorimeter.

The whole procedure was done in triplicate for both strains (using different culture batches) to verify the obtained results. In order to adjust the initial viable bacterial concentration to measurable levels of fluorescence intensity, a correlation between viable cell fluorescence intensity and plate counts was established as follows. Cell suspensions were diluted in ultrapure water and plated (in duplicate) on MRS/TJ medium with 2.0% (w/v) agar. Simultaneously, the viable cell fluorescence of the suspension was measured as described above. All plates were incubated at 25 °C for 7 days. The results have shown a good correlation between viable cell fluorescence and plate counts for both strains ( $r^2 = 0.98$  and  $r^2 = 0.99$  for *O. oeni* VF and *L. hilgardii* 5, respectively).

#### Statistical analysis

Since it is generally assumed that ion fluxes across the cellular membrane occur by passive diffusion and normally follow first-order kinetics (da Silveira et al., 2003; Gilbert, 1984), we have calculated first-order kinetic models for these ion fluxes using Origin software version 6.1 from OriginLab (Northampton, MA). Overall, a good fitting was obtained with these models (Tables 1–3).

In the potassium efflux experiments, the maximum extracellular potassium concentration (*A*) and the efflux rate (*k*) were calculated

**Table 1**

Variation of the extracellular K<sup>+</sup> concentration of lactic acid bacteria cell suspensions exposed to different phenolic acids (at 2.2 g/L) in phosphate buffer with 11% (v/v) ethanol.

Phenolic acid	<i>Oenococcus oeni</i> VF			<i>Lactobacillus hilgardii</i> 5		
	A* mM/g	k* (×10 <sup>-3</sup> /s)	r <sup>2</sup> *	A* mM/g	k* (×10 <sup>-3</sup> /s)	r <sup>2</sup> *
(Control)	2.3 ± 0.2 <sup>a</sup>	44.8 ± 15.4 <sup>a</sup>	0.889	1.4 ± 0.2 <sup>a</sup>	55.2 ± 22.8 <sup>a</sup>	0.837
Gallic	3.4 ± 0.2 <sup>b</sup>	32.9 ± 7.4 <sup>a</sup>	0.907	1.6 ± 0.2 <sup>b</sup>	34.1 ± 13.6 <sup>ab</sup>	0.855
Protocatechuic	3.0 ± 0.3 <sup>c</sup>	29.2 ± 7.9 <sup>bcd</sup>	0.870	1.7 ± 0.2 <sup>bc</sup>	34.9 ± 11.1 <sup>abc</sup>	0.819
Vanillic	3.1 ± 0.3 <sup>c</sup>	14.2 ± 3.3 <sup>e</sup>	0.920	1.8 ± 0.2 <sup>cd</sup>	14.5 ± 3.9 <sup>d</sup>	0.885
p-Coumaric	5.0 ± 0.5 <sup>d</sup>	8.9 ± 2.0 <sup>f</sup>	0.949	4.6 ± 0.5 <sup>e</sup>	9.4 ± 2.3 <sup>e</sup>	0.936
p-Hydroxybenzoic	2.1 ± 0.2 <sup>ae</sup>	27.1 ± 7.4 <sup>bc</sup>	0.865	1.3 ± 0.1 <sup>af</sup>	33.4 ± 10.1 <sup>ab</sup>	0.831
Ferulic	4.2 ± 0.3 <sup>f</sup>	22.6 ± 5.5 <sup>dg</sup>	0.899	1.9 ± 0.2 <sup>d</sup>	24.6 ± 6.8 <sup>bcd</sup>	0.867
Caffeic	3.7 ± 0.3 <sup>g</sup>	21.6 ± 5.8 <sup>g</sup>	0.874	2.2 ± 0.2 <sup>g</sup>	22.5 ± 6.9 <sup>bf</sup>	0.835
Syringic	2.0 ± 0.2 <sup>e</sup>	34.0 ± 9.3 <sup>abc</sup>	0.865	1.5 ± 0.2 <sup>abf</sup>	46.4 ± 16.0 <sup>abc</sup>	0.776

\*Parameters obtained after first-order model fittings —  $C(t) = A(1 - e^{-k \cdot t})$ : A — maximum extracellular potassium concentration; k — potassium efflux rate; r<sup>2</sup> — coefficient of determination (goodness-of-fit) of the statistical model. Concentration values are expressed per wet weight of cellular pellet. Values with different superscript letters represent significant differences (p < 0.05) and errors represent standard deviations of the parameters.

from the first-order equation  $C(t) = A(1 - e^{-k \cdot t})$  obtained after fitting. The same approach was used to estimate the same parameters in the phosphate efflux experiments.

Total proton influx was calculated from first-order fittings using the equation  $C(t) = C_0 + A \cdot e^{k \cdot t}$ , where C<sub>0</sub> represents the extracellular concentration of H<sup>+</sup> immediately after the addition of the phenolic acids, A represents the asymptotical value of the extracellular concentration of H<sup>+</sup> and k' represents the proton efflux rate. Since C<sub>0</sub> was not the same in all experiments (due to differences in the pK<sub>a</sub>s of the various phenolic acids), we have calculated the variation of extracellular H<sup>+</sup> concentration (ΔC) after 5 min of exposure from the model equations in order to compare the effect of the different phenolic acids.

The obtained model parameters were compared using two-sided F-tests and Student's t-tests. Analyses of variance (ANOVA) and Tukey's HSD (Honestly Significant Differences) were used to compare fluorescence intensity values between the different assays using Statistica for Windows version 4.5 from Statsoft (Tulsa, OK). All statistical tests were performed at a confidence level of 95% (p = 0.05).

## Results

Hydroxycinnamic acids (p-coumaric, caffeic and ferulic acids) caused higher potassium efflux than hydroxybenzoic acids in both strains of bacteria tested (Fig. 2, Table 1). p-Coumaric acid had the highest effect of all tested phenolic acids against both bacterial strains.

In the case of *O. oeni* VF, p-hydroxybenzoic and syringic acids did not cause an increased potassium efflux comparatively to the control (Fig. 2, Table 1). The phenolic acids which had a significant effect on

the total potassium efflux were (in decreasing order): p-coumaric > ferulic > caffeic > gallic > protocatechuic and vanillic acids.

In the experiments performed with *L. hilgardii* 5, an extensive potassium efflux was found in the presence of p-coumaric acid, comparatively with the control (Fig. 2b, Table 1). The compounds which had a greater effect on total potassium efflux were (in decreasing order): p-coumaric > caffeic > ferulic and vanillic > protocatechuic and gallic acids. p-Hydroxybenzoic and syringic acids did not have a significant effect on the total potassium efflux, comparatively to the control.

Comparing the maximum (initial) efflux rates, we observe that generally, the addition of phenolic acids to the suspending medium resulted in a decrease in the efflux rate in comparison to the control (Table 1). In *O. oeni* VF, most phenolic acids (except gallic and syringic acids) affected the potassium efflux rate while in *L. hilgardii* 5, the three hydroxycinnamic acids and vanillic acid had significantly lower values than the control (Table 1).

Following the addition of the phenolic acids to the cell suspensions, a quick increase in extracellular proton concentration occurred followed by a steady decrease presumably due to proton influx (Fig. 3). Comparing the behaviour of cells exposed to the different phenolic acids, it can be observed that p-coumaric again had the strongest effect of all tested acids in both strains (Fig. 3, Table 2). In the experiments with *O. oeni* VF all phenolic acids, except vanillic acid, caused a significant total proton influx while in the *L. hilgardii* 5 experiments, p-coumaric had a much stronger effect than the other phenolic compounds (Table 2). The first-order kinetics model used did not fit well the data obtained in the syringic acid experiments. However, the variation of extracellular H<sup>+</sup> concentration after 5 min of exposure was relatively low and similar to the levels obtained in the control assays: — 5.9 μM/g

**Table 2**

Variation of the extracellular H<sup>+</sup> concentration of lactic acid bacteria cell suspensions exposed to different phenolic acids (at 2.2 g/L) in phosphate buffer with 11% v/v ethanol.

Phenolic acid	<i>Oenococcus oeni</i> VF			<i>Lactobacillus hilgardii</i> 5		
	ΔC* (μM/g)	k* (×10 <sup>-3</sup> /s)	r <sup>2</sup> *	ΔC* (μM/g)	k* (×10 <sup>-3</sup> /s)	r <sup>2</sup> *
(Control)	−9.7 ± 1.7 <sup>a</sup>	−11.9 ± 2.9 <sup>a</sup>	0.964	−6.6 ± 0.8 <sup>a</sup>	−8.3 ± 1.5 <sup>a</sup>	0.988
Gallic	−35 ± 6 <sup>b</sup>	−6.7 ± 1.2 <sup>b</sup>	0.990	−17 ± 3 <sup>b</sup>	−6.7 ± 1.2 <sup>ab</sup>	0.990
Protocatechuic	−26 ± 2 <sup>bc</sup>	−3.5 ± 1.3 <sup>cd</sup>	0.988	−13 ± 9 <sup>ab</sup>	−3.5 ± 1.3 <sup>ce</sup>	0.988
Vanillic	−16 ± 7 <sup>ac</sup>	−5.3 ± 1.7 <sup>bc</sup>	0.979	−8.0 ± 3.7 <sup>ac</sup>	−5.3 ± 1.7 <sup>bcd</sup>	0.979
p-Coumaric	−55 ± 18 <sup>d</sup>	−3.8 ± 1.1 <sup>cd</sup>	0.989	−54 ± 7 <sup>d</sup>	−4.9 ± 0.6 <sup>df</sup>	0.998
p-Hydroxybenzoic	−32 ± 18 <sup>bc</sup>	−3.3 ± 1.2 <sup>d</sup>	0.989	−18 ± 15 <sup>ab</sup>	−3.1 ± 1.4 <sup>eg</sup>	0.986
Ferulic	−34 ± 12 <sup>bc</sup>	−3.8 ± 0.8 <sup>cd</sup>	0.994	−15 ± 3 <sup>b</sup>	−4.5 ± 0.6 <sup>cdf</sup>	0.998
Caffeic	−29 ± 11 <sup>bc</sup>	−4.4 ± 1.3 <sup>cd</sup>	0.988	−15 ± 7 <sup>bc</sup>	−4.4 ± 1.3 <sup>cdg</sup>	0.988

\* Parameters obtained after first-order model fittings —  $C(t) = C_0 + A \cdot e^{k \cdot t}$ : ΔC — variation of extracellular H<sup>+</sup> concentration after 5 min of exposure (calculated from the model equations); k' — proton efflux rate; r<sup>2</sup> — coefficient of determination (goodness-of-fit) of the statistical model. All concentration values are expressed per wet weight of cellular pellet. Values with different superscript letters represent significant differences (p < 0.05). Errors represent standard deviations of the parameters (n = 10). Syringic acid data is not shown due to poor fitting of the model.



**Table 3**Potassium efflux and proton influx of *O. oeni* VF cell suspensions exposed to different concentrations of *p*-coumaric acid in phosphate buffer with 11% v/v ethanol.

[ <i>p</i> -Coumaric acid] (g/L)	Potassium (K <sup>+</sup> ) efflux			Proton (H <sup>+</sup> ) influx		
	A* mM/g	k* (×10 <sup>-3</sup> /s)	r <sup>2</sup> *	ΔC** mM/g	k'*** (×10 <sup>-3</sup> /s)	r <sup>2</sup> **
0	0.7 ± 0.1 <sup>a</sup>	14.9 ± 1.1 <sup>a</sup>	0.990	-3.7 ± 0.5	-10.3 ± 2.4 <sup>a</sup>	0.970
0.69	3.3 ± 0.2 <sup>b</sup>	6.2 ± 0.5 <sup>b</sup>	0.999	-29 ± 5	-4.3 ± 0.6 <sup>b</sup>	0.998
1.4	3.2 ± 0.1 <sup>b</sup>	6.7 ± 0.5 <sup>b</sup>	0.996	-25 ± 2	-6.2 ± 0.6 <sup>c</sup>	0.998
2.8	4.9 ± 0.1 <sup>c</sup>	11.4 ± 0.4 <sup>c</sup>	0.997	-56 ± 3	-9.2 ± 0.6 <sup>a</sup>	0.998
5.6	4.0 ± 0.1 <sup>d</sup>	67.3 ± 4.2 <sup>d</sup>	0.989	-130 ± 6	-42.3 ± 3.8 <sup>d</sup>	0.993

\*Potassium efflux parameters obtained after first-order model fittings –  $C(t) = A(1 - e^{-kt})$ : A – maximum extracellular potassium concentration; k – potassium efflux rate; r<sup>2</sup> – coefficient of determination (goodness-of-fit) of the statistical model.

\*\*Proton influx parameters obtained using first-order model fittings –  $C(t) = C_0 + A \cdot e^{k' \cdot t}$ ; k' – proton influx rate; ΔC – variation of extracellular H<sup>+</sup> concentration after 5 min of exposure (calculated from the model equations); r<sup>2</sup> – coefficient of determination (goodness-of-fit) of the statistical model.

Concentration values are expressed per wet weight of cellular pellet. Values with different superscript letters represent significant differences (p < 0.05) and errors represent standard deviations of the parameters.

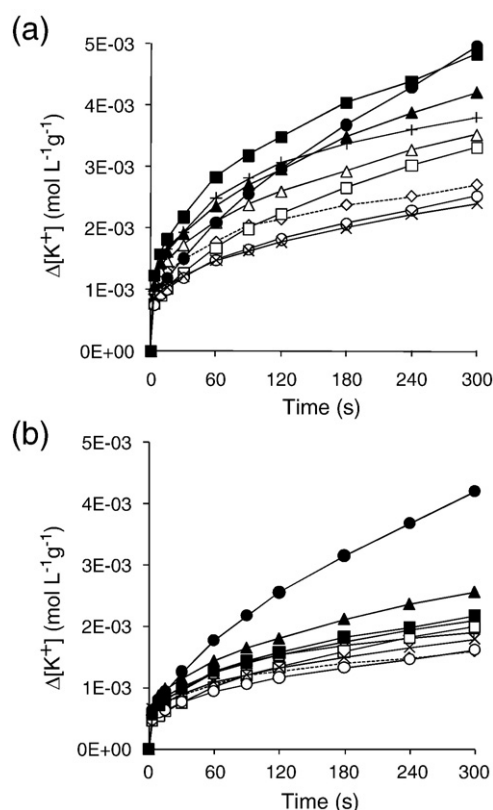
for *O. oeni* VF and -4.4 μM/g for *L. hilgardii* 5. Most phenolic acids decreased the proton influx rate comparatively to the control (Table 2). However, this effect was more noticeable in *O. oeni* VF than in *L. hilgardii* 5.

The exposure of *O. oeni* VF cell suspensions to increasing levels of *p*-coumaric acid caused a high potassium efflux and proton influx (Fig. 4, Table 3). A significant decrease in potassium efflux rate (and an increase in proton influx rate) was observed at concentrations of *p*-coumaric acid up to 2.8 g/L, despite the total potassium efflux being higher than the control (Table 3).

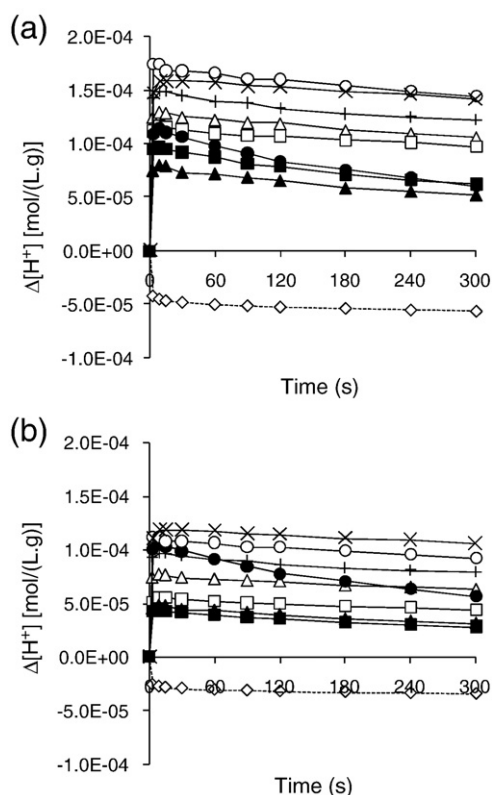
At the highest concentration tested of *p*-coumaric acid, however, both potassium efflux and proton influx rates were higher than the control (Table 3). The rapid ion fluxes observed at higher concentrations of *p*-coumaric acid might be correlated with the previously

observed loss of viability of *O. oeni* VF at high concentration levels of this compound (Campos et al., 2003). Similar statistically significant results were obtained with *L. hilgardii* 5 suspensions, though the effect of *p*-coumaric acid was weaker than in *O. oeni* VF, at the same concentration levels of this phenolic acid (results not shown).

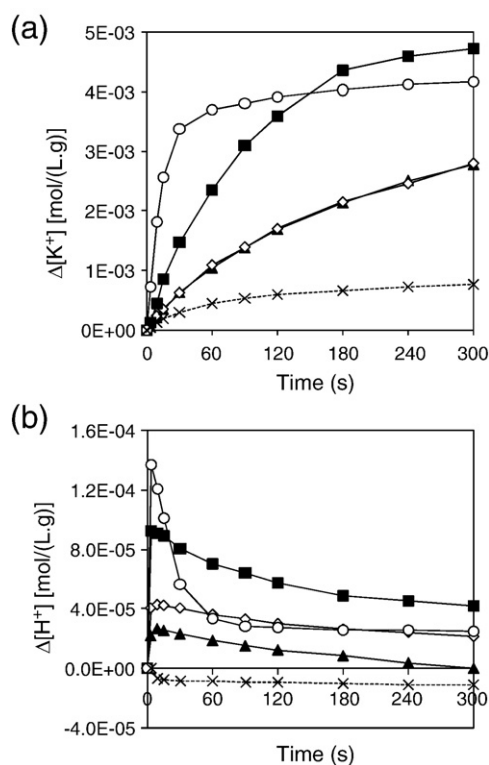
Moreover, the addition of an organic (L-lactic acid) and inorganic (hydrochloric acid) acids to the suspending medium at a similar molar concentration (15 mM) did not cause enhanced potassium efflux from the bacterial suspensions (results not shown). This observation suggests that the increased potassium efflux observed in the presence of phenolic acids was not due to the sudden acidification of the extracellular suspending medium or to the acidification of the cytoplasm by the intracellular dissociation of organic acids.



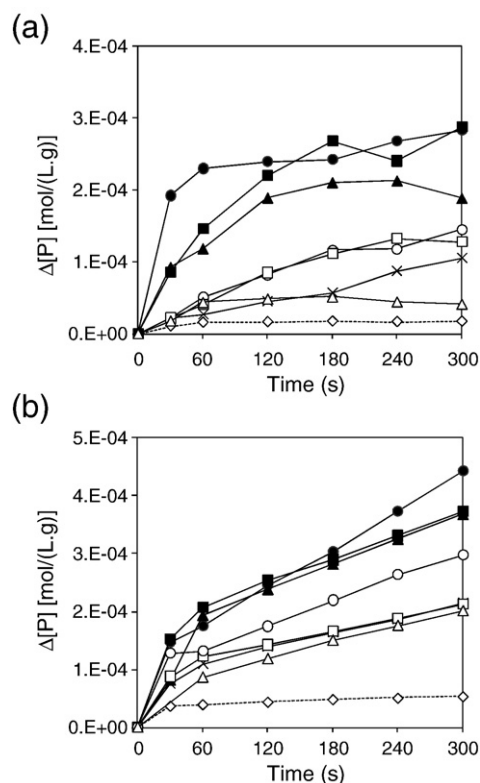
**Fig. 2.** Potassium efflux of (a) *Oenococcus oeni* VF and (b) *Lactobacillus hilgardii* 5 suspensions exposed to chemical stress with different phenolic acids (at 2.2 g/L) in phosphate buffer with 11% v/v ethanol: (●) *p*-coumaric acid, (▲) caffeic acid, (■) ferulic acid, (○) *p*-hydroxybenzoic acid, (△) protocatechuic acid, (□) vanillic acid, (+) gallic acid, (x) syringic acid, (◇) control; values are expressed as variation of extracellular K<sup>+</sup> concentration per wet weight of cellular pellet.



**Fig. 3.** Proton influx of (a) *Oenococcus oeni* VF and (b) *Lactobacillus hilgardii* 5 suspensions exposed to chemical stress with different phenolic acids (at 2.2 g/L) in phosphate buffer with 11% (v/v) ethanol: (●) *p*-coumaric acid, (▲) caffeic acid, (■) ferulic acid, (○) *p*-hydroxybenzoic acid, (△) protocatechuic acid, (□) vanillic acid, (+) gallic acid, (x) syringic acid, (◇) control; values are expressed as variation of extracellular H<sup>+</sup> concentration per wet weight of cellular pellet.



**Fig. 4.** Variation of the extracellular concentrations of potassium (a) and hydrogen (b) ions of *Oenococcus oeni* VF suspensions exposed to different concentrations of p-coumaric acid (in phosphate buffer with 11% v/v ethanol): (x) control, (▲) 0.69 g/L, (◇) 1.4 g/L, (■) 2.8 g/L, (○) 5.6 g/L; results are expressed per wet weight of cellular pellet.



**Fig. 5.** Phosphate efflux of (a) *Oenococcus oeni* VF and (b) *Lactobacillus hilgardii* 5 suspensions exposed to chemical stress with different phenolic acids (at 2.2 g/L) in ultrapure water with 11% v/v ethanol: (●) p-coumaric acid, (▲) caffeic acid, (■) ferulic acid, (○) p-hydroxybenzoic acid, (△) protocatechuic acid, (□) vanillic acid, (x) syringic acid, (◇) control; results are presented as variation of phosphate concentration (expressed in molar phosphorus concentration) per wet weight of cellular pellet.

Total phosphate efflux of *O. oeni* VF suspensions was significantly increased by the presence of all phenolic acids (Fig. 5a, Table 4). Again, this effect was generally greater in the case of the hydroxycinnamic acids than in the case of hydroxybenzoic acids. The order of the effect was (in decreasing order): ferulic > p-coumaric > caffeic > p-hydroxybenzoic and vanillic > protocatechuic acid. Syringic acid results did not fit well the first-order statistical model. However, the observed phosphate efflux was relatively low (0.10 mM/g). The highest phosphate efflux rate was observed in the p-coumaric acid assay despite the difference with respect to the control was not statistically significant. All other phenolic compounds decreased the phosphate efflux rate comparatively to the control (Table 4).

Similar results were obtained in the experiments with *L. hilgardii* 5 (Fig. 5b, Table 4), with all phenolic acids causing an increased total phosphate efflux and decreased efflux rate (relative to the control). Hydroxycinnamic acids (and p-coumaric acid in particular) caused the largest efflux. The observed order of the effect was p-coumaric > caffeic and ferulic > p-hydroxybenzoic > protocatechuic > syringic and vanillic acids.

All phenolic acids caused a significant decrease in viable cell fluorescence of *O. oeni* VF and *L. hilgardii* 5, relative to the controls. In the experiments done with *O. oeni*, the observed decrease in fluorescence intensity varied between 44% (in the caffeic acid assay) and 59% (in the p-coumaric acid assay); in *L. hilgardii*, the highest fluorescence decrease was observed in the p-coumaric acid assay (77%) and the lowest, in the gallic acid assay (63%). Despite p-coumaric acid caused the highest effect in both tested strains, it was not possible to statistically differentiate it from that of the other phenolic acids.

In the experiments with *L. hilgardii* 5, we found that increasing concentrations of both p-coumaric acid and gallic acid increasingly reduced the viable cell fluorescence intensity of this strain after 10 min of exposure to these compounds. Resting-cell suspensions of *L. hilgardii* exposed to gallic acid at concentration levels of 250, 500 and 1000 mg/L caused a reduction of viable cell fluorescence of 67%, 81% and 87%, respectively; the same concentrations of p-coumaric acid caused a decrease of 60%, 88% and 97%, respectively.

## Discussion

Phenolic acids are thought to act both at the membrane and cytoplasmic levels, the latter mechanism being considered to be due to reducing intracellular pH and acting as protoplasmic poisons.

Phenolic acids are weak organic acids ( $pK_a \sim 4.2$ ) and their antimicrobial activity is dependent on the concentration of the undissociated acid. Due to their partially lipophilic nature, it is assumed that phenolic acids cross the cell membrane by passive diffusion in their undissociated form, disturbing the cell membrane structure and possibly acidifying the cytoplasm and causing protein denaturation.

The primary action of phenolic acids on the cell membrane has been supported by several published works. Ramos-Nino et al. (1996) found that the antilisterial activity of phenolic acids was dependent on the lipophilicity and degree of ionization of the molecule. Other authors also found the phenolic acid activity against *Listeria monocytogenes* to be dependent on pH, which is consistent with the passive diffusion of phenolic acids through the cell membrane (Kouassi and Shelef, 1998; Wen et al., 2003).

The observed potassium effluxes in the tested strains were found to be related to the specific chemical structure of phenolic acids rather than to an acid shock or to the intracellular acidification caused by organic acids.

Wine lactic bacteria are known to possess adaptation mechanisms to counteract damage caused by ethanol by increasing membrane fluidity (Couto et al., 1996, 1997; da Silveira et al., 2003; Teixeira et al., 2002). It has also been demonstrated that some LAB strains may respond to the presence of phenolic acids by increasing the unsaturated fatty acid content (and presumably the fluidity) of the cell

**Table 4**

Phosphate efflux of lactic acid bacteria cell suspensions exposed to different phenolic acids (at 2.2 g/L) in phosphate buffer with 11% v/v ethanol.

Phenolic acid	<i>Oenococcus oeni</i> VF			<i>Lactobacillus hilgardii</i> 5		
	A* mM/g	k* ( $\times 10^{-3}$ /s)	r <sup>2</sup> *	A* mM/g	k* ( $\times 10^{-3}$ /s)	r <sup>2</sup> *
(Control)	0.02 $\pm$ 3E-4 <sup>a</sup>	35.4 $\pm$ 2.3 <sup>a</sup>	0.996	0.05 $\pm$ 3E-4 <sup>a</sup>	38.3 $\pm$ 8.7 <sup>a</sup>	0.958
Protocatechuic	0.05 $\pm$ 4E-4 <sup>b</sup>	25.8 $\pm$ 7.9 <sup>b</sup>	0.919	0.23 $\pm$ 0.02 <sup>b</sup>	6.5 $\pm$ 1.2 <sup>b</sup>	0.989
Vanillic	0.17 $\pm$ 0.02 <sup>c</sup>	5.7 $\pm$ 1.3 <sup>c</sup>	0.986	0.19 $\pm$ 0.01 <sup>c</sup>	15.0 $\pm$ 3.3 <sup>c</sup>	0.955
p-Coumaric	0.26 $\pm$ 0.01 <sup>d</sup>	42.5 $\pm$ 7.7 <sup>a</sup>	0.976	0.47 $\pm$ 0.07 <sup>d</sup>	6.8 $\pm$ 2.2 <sup>d</sup>	0.954
p-Hydroxybenzoic	0.18 $\pm$ 0.02 <sup>c</sup>	5.3 $\pm$ 1.1 <sup>c</sup>	0.990	0.29 $\pm$ 0.04 <sup>e</sup>	9.9 $\pm$ 3.3 <sup>e</sup>	0.922
Ferulic	0.28 $\pm$ 0.01 <sup>e</sup>	12.4 $\pm$ 1.9 <sup>d</sup>	0.983	0.34 $\pm$ 0.02 <sup>f</sup>	14.5 $\pm$ 3.1 <sup>f</sup>	0.959
Caffeic	0.21 $\pm$ 0.01 <sup>ef</sup>	17.4 $\pm$ 2.8 <sup>e</sup>	0.976	0.37 $\pm$ 0.03 <sup>f</sup>	9.4 $\pm$ 1.8 <sup>f</sup>	0.978
Syringic	—**	—	—	0.20 $\pm$ 0.01 <sup>c</sup>	11.5 $\pm$ 2.3 <sup>c</sup>	0.969

\*Parameters obtained after first-order model fittings —  $C(t) = A(1 - e^{-kt})$ : A — maximum extracellular phosphate concentration; k — phosphate efflux rate (values expressed per wet weight of cellular pellet); r<sup>2</sup> — coefficient of determination of the statistical model. Values with different superscript letters represent significant differences ( $p < 0.05$ ). Errors represent standard deviations of the parameters.

\*\* Syringic acid data for *O. oeni* VF is not shown due to poor fitting of the model.

membrane (Rozès and Peres, 1998). However, under resting-cell conditions, bacteria might not be able to counteract the combined effect of phenolic acids and ethanol on the cytoplasmic membrane.

In chemically-induced cell injury, the effects depended on the biocide concentration/cell number ratio, rather than on the absolute concentration of the biocide (Gilbert, 1984). Therefore, it can be assumed that the results obtained using concentrated cell suspensions and concentrated phenolic acid solutions may be comparable to the results obtained at lower concentrations of both parameters. The results obtained in this work with the ion efflux monitoring systems agree, in general, with previous growth experiments with the same strains and the same phenolic acid concentration: /bacterial concentration ratio (Campos et al., 2003). Total ion effluxes were higher in the case of the phenolic acids which had a more negative effect on the growth of these bacteria. On the other hand, inactivation results obtained in the same work did not seem to correlate entirely with the measured ion effluxes which may indicate that the membrane damage caused by phenolic acids may be reversible if cells are subsequently transferred to a growth medium or that bacterial inactivation by phenolic acids might involve more than one mechanism or cellular target. The viable fluorescence results were not entirely conclusive since no statistically significant differences were observed between the individual effects of the various phenolic acids tested. In a previous work, other authors (da Silveira et al., 2002) using flow cytometric analysis, found large sub-populations of double-stained *O. oeni* cells with fluorescent dyes following exposure to ethanol. Thus, incomplete dye exclusion might have affected the direct fluorescence measurements. Nevertheless, the fluorescence experiments confirmed that all phenolic acids increased cell membrane permeability as indicated by a reduction in the overall viable cell fluorescence.

The strains used in this work were previously found to be unable to decarboxylate p-coumaric and ferulic acids to the corresponding vinylphenols (Couto et al., 2006). Therefore, the possibility of an increased proton influx and potassium efflux due to the active decarboxylation of these acids does not seem feasible in these strains.

To our knowledge, this is the first report of the effect of wine phenolic compounds on wine-associated LAB. Moreover, different instrumental techniques were used to evaluate these effects, which reinforce our findings.

Altogether, the results indicate that most of the phenolic acids (and hydroxycinnamic acids in particular) tested increased the cell membrane permeability of the LAB strains studied. The different effects could be related to differences in their structure and lipophilic character.

Though the concentrations of phenolic acids used in this work were greater than those normally found in wines, it should be pointed out that lower pHs found in wines may facilitate the diffusion of phenolic acids through the cytoplasmic membrane and also that other antimicrobial agents may have synergistic effects with these com-

pounds on the bacterial membrane. The effects of phenolic acids on the cell membrane of LAB need to be further investigated in more approximate conditions to wine.

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